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Applicant : Johan Weigelt et al.
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Art Unit : 1642
Examiner :

Commissioner for Patents
Washington, D.C. 20231

TRANSMITTAL OF CERTIFIED PRIORITY DOCUMENT UNDER 35 USC §119

In accordance with the provisions of 35 U.S.C. §119, applicants hereby claim priority of Swedish Patent application No. 0003811-7, filed October 20, 2000. A certified copy of the application is submitted herewith. As the priority application is in the English language, all of the requirements of §119 have been met.

Please apply any charges or credits to Deposit Account No. 06-1050, referencing Attorney Docket No. 13425-047001.

Respectfully submitted,

Date: February 27, 2002

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SCREENING METHODS

TECHNICAL FIELD

The present invention relates to a nuclear magnetic resonance (NMR) based method for assaying binding of various chemical compounds to a polypeptide or a protein. More specifically, the method allows for the screening of binding to a designated binding epitope on the surface of the polypeptide or protein.

BACKGROUND ART

In modern biology and medicine, there has been a demand for physical methods, which can make it possible to study the structure of small and large biomolecules, as well as the interaction between various molecules and compounds. For this purpose, several powerful techniques have been applied, such as x-ray crystallography, mass spectrometry, and nuclear magnetic resonance (NMR).

In recent years, NMR spectroscopy has become an important tool in the drug discovery process through the advent of NMR based screening methods to identify lead templates. Several techniques have been introduced, [Shuker, 1996; Chen, 1998; Mayer, 1999; Chen, 2000; Jahnke, 2000] perhaps the most well known is the "SAR by NMR" method described by Fesik and coworkers in 1996. [Shuker, 1996 and EP-B1-0866967] The SAR by NMR technique relies on detecting chemical shift changes in a two-dimensional ^{1}H-^{15}N correlation spectrum to identify compounds that bind to the target protein. When a first ligand has been identified, a second ligand is sought for in the presence of saturating concentrations of the first ligand (screen for "second-site" binder). Provided the three dimensional structure of the protein is known and sequence specific NMR assignments of the protein backbone resonances have been obtained, the two ligands may be linked based on structural data. To first approximation the binding affinity of the linked compound will be the product of the binding affinities of the individual compounds, resulting in high affinity "nanomolar binders" even if the starting compounds only had millimolar to micromolar affinities. [Shuker, 1996]

A prerequisite for the SAR by NMR method is that sequence specific resonance assignments have been obtained for the backbone NMR resonances (^{15}N, $^{13}C_{\alpha}$, $^{13}C'$, $^{1}H^{N}$) of the target protein. This is a formidable task that demands several months of experimental work and data analysis even for a relatively small protein.

Thus, there is a need for a method allowing identification of binder molecules to a specific target in an easier and more effective way, thereby limiting the experimental work.

The object of the invention is to reduce the drawbacks and expand the possibilities of the prior art.

SUMMARY OF THE INVENTION

This object is, according to the invention, solved by a method for identifying at least one binder molecule comprising the steps of:

(a) choosing two amino acid types (AA1 and AA2) in a polypeptide or protein of interest, whereby AA2 at least once occurs directly subsequent to AA1 in the amino acid sequence of the polypeptide or protein, defining an amino acid pair AA1-AA2;

(b) labelling the two amino acid types (AA1 and AA2) in the polypeptide or protein of interest, whereby all AA1-residues is labelled with ^{13}C and all AA2-residues with ^{15}N;

(c) generating a first HNCO-type NMR spectrum of the labelled polypeptide or protein from step (b), thereby identifying signals from the labelled amino acid pair AA1-AA2;

(d) contacting the labelled polypeptide or protein with a potential binder molecule or a mixture of binder molecules under conditions and sufficient time for allowing binding of the potential binder molecule(s) and the labelled polypeptide or protein;

(e) generating a second HNCO-type NMR spectrum, or a ^{1}H-^{15}N correlation type NMR spectrum, of the mix from step (d), monitoring signals identified in step (c);

(f) comparing the first and the second NMR spectra, whereby a chemical shift change of the signals identified in step (c) between the two spectra indicates an interaction between the potential binder molecule and the labelled polypeptide or protein.

Yet another aspect of the invention is a method, whereby the labelled amino acid pair AA1-AA2 is unique within a sphere radius of 10 Å, preferably 50 Å, and most preferably within the whole polypeptide or protein.

One embodiment of the invention is a method, whereby the labelled amino acid pair AA1-AA2 is within a binding pocket of the polypeptide or protein. This allows for the screening for binding to a single binding site. The requirement is that a unique amino acid pair occurs in the binding pocket.

Another embodiment of the invention is a method, whereby the labelled amino acid pair AA1-AA2 is in the proximity, preferably closer than 15 Å, of an active site within the polypeptide or protein. This enables the screening for specificity in vicinity of an active site in the polypeptide or protein. If the target protein has one or more unique amino acid pairs in the vicinity of an active site, whereby the unique amino acid pairs differs from other members of the same protein family, this can be used to screen for selectivity, i.e. the different proteins of the same family can be compared. Different compounds may give different chemical shift, why the longest distance from the active site, which makes specificity screening possible, varies.

Yet another embodiment of the invention is a method, whereby the result of the method is compared to the result of any other suitable binding or activity assay, such as a fluorescence-based assay, a reporter gene assay, displacement assays or ELISA. This allows for a rapid confirmation of the binding to this specifically labelled site, or as a selection of candidates for more extensive study by any other suitable method.

Accordingly, according to another aspect of the invention, the method is used for screening of a compound library.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the site specific labelling of the PTP1B-protein.

Figure 2 shows 2D NMR spectra of ^{15}N-Asp, ^{13}C-Arg labelled PTP1B.

Figure 3 is a ribbon drawing of PTP1B showing the labelled amino acid residue pairs.

Figure 4 is a table showing the result of a colorimetric assay.

Figure 5 is a stereo-view of PNU179983 binding to PTP1B.

Figure 6 is a schematic view of one embodiment of the invention (screening for binding to a single binding site).

Figure 7 is a schematic view of another embodiment of the invention (screening for specificity in vicinity of an active site).

DISCLOSURE OF THE INVENTION

By a "compound library" is meant a set of chemical compounds, that for example can be used for assaying binding to a macromolecule. The compound library may comprise from about 2 – 100.000 compounds, preferably 100-1000. The compounds of the library can be of any size, preferably 50-1000 D. The compounds can be any organic molecules or natural products.

By a "polypeptide or protein of interest" is meant any amino acid sequence, or a complex of amino acid sequences, having a potential binding epitope.

By a "potential binder molecule" is meant a chemical compound, which may bind to the polypeptide or protein of interest, and which may be a member of the compound library. The potential binder molecule may be a peptide, a polypeptide, a protein, an antibody, a

nucleic acid molecule, a carbohydrate, or a part or a complex of one or more of the mentioned molecule types, or any other chemical compound of interest. Preferably, the potential binder molecule is a relatively small molecule, such as a molecule in the size interval of 50-1000 D.

"AA1" and "AA2" can be any of the 20 different amino acid types. AA1 and AA2 may be the same or different type.

By AA2 at least once occurs "directly subsequent" to AA1 in the amino acid sequence is meant that AA1 in this case has the sequence position n-1 and AA2 sequence position n.

A "AA1-AA2-pair" is a pair of amino acids following each other directly in sequence, whereby AA1 has the number n-1 and AA2 the number n in the amino acid sequence.

By a "binding pocket" is meant an epitope on the polypeptide or protein of interest, that is expected to allow binding of a potential binder molecule.

By a "sphere radius" in this context is meant a defined distance in any direction in space from a defined pair of amino acids (AA1-AA2).

The core of the invention is a revitalization of a sequence specific labelling method that has not been extensively used. [Kainosho, 1982][Kato, 1991] All amino acids AA1 are labelled with ^{13}C and all amino acids AA2 are labelled with ^{15}N. Provided only one AA1-AA2 pair occurs in the amino acid sequence, only one signal in the 1D carbonyl ^{13}C spectrum will display a splitting due to the $^{1}J_{CN}$ coupling. Obviously only one peak will appear in a 1D (or 2D or 3D) HNCO type correlation spectrum (Figure 1).

The labelling strategy is only sequence specific in an indirect sense, *i.e.* the occurrence of a unique pair of labelled amino acid residues confers the sequence specificity. Using this technique it is possible to screen selectively for binding to a selected epitope without the need for sequence specific assignments. The HNCO spectrum can thus be used either directly as a screening experiment (1D or 2D versions) or indirectly to

identify what signals to monitor in a 2D ^{1}H-^{15}N correlation spectrum. Chemical shift perturbations upon addition of a potential ligand are easily detected even for large proteins due to the reduced spectral complexity resulting from the use of a selectively labelled sample.

The site specificity is inherent to the labelling technique and the key is to find a unique sequence motif in the binding site that is to be screened. Given a protein of 300 amino acid residues, and assuming equal and random distribution of all 20 amino acid residues, the probability that a given amino acid residue pair only occurs once in the sequence is approximately 0.5. If the desired site contains at least 3-4 suitable pairs, the probability of finding a unique pair is reasonably high, *i.e.* 85-95 %.

The most reliable way to obtain a sequence specific labelled protein is to over-express the protein in rich medium containing the labelled amino acids, using a bacterial strain with gene lesions suitable for the particular type of selective amino acid enrichment. [Muchmore, 1989] In favorable cases however, good results have been reported using a prototrophic bacterial strain in a rich medium containing all amino acids and nucleotides.[Kainosho, 1982] [Muchmore, 1989]

According to one embodiment of the invention, the method can be used for screening for binding to a single binding site (Fig. 6). This can be done if the target protein or polypeptide of interest has at least one unique amino acid pair (two amino acids being adjacent to each other in the primary structure) within the potential binding site. Then, this unique amino acid pair is labelled according to the method of the invention, and the interaction between the binding site and potential binder molecules can be studied.

According to another embodiment of the invention, the method can be used for screening for specificity in vicinity of an active site (Fig. 7). This can be achieved if the target protein or polypeptide of interest has at least one unique amino acid pair in vicinity of the active site (preferably closer than 15 Å, and more preferably within 5-15 Å). Further, if the target protein differs from other proteins within the same protein family in that the target protein has at least one unique amino acid compared to the other proteins, this can be used to screen for selectivity of potential binder molecules to

the target protein. This means that the unique amino acid, which differs from the other proteins of the family, may be close to the active site with regard to tertiary structure, but not necessarily close with regard to primary structure. However, the unique amino acid should preferably not be within the active site.

According to yet another embodiment of the invention, the method of the invention can be used in combination with any other suitable binding or activity assay, such as a fluorescence based assay, a reporter gene assay, displacement assays or ELISA, in order to either confirm the result of that method, or to screen for interesting compounds, which subsequently are more thoroughly studied by another method. The method of the invention can be advantageously used in this aspect as it provides a method, which rapidly gives a reliable result. This can be useful when there are no X-ray data for the studied protein, but only computer modelling data.

The conditions suitable for allowing the potential binder molecule and the labelled protein or polypeptide of interest to interact, as well as to monitor the interaction by NMR, are standard conditions for protein NMR [Cavanagh] (buffered solutions, pH kept stable, reaction temperature 5-50°C). Preferably, the target protein concentration may range from 25 μM to 1 mM, and the potential binder molecule concentration from 25 μM to 1 mM.

In order for a change in the chemical shift to be considered relevant, the change should be equal to or greater than the natural line width, or the signals should be exchange-broadened beyond detection.

Moreover, the method of the invention may be used for competition binding analysis experiments.

To summarise, the new technique presents a new approach to screen and identify binders to protein targets in a site-specific manner. This will allow the identification of scaffolds (by screening a small compound library) with a binding preference for a particular site or sites, which could confer specificity for a certain target within a protein family, and also confirm the binding mode from hits detected using other suitable

binding or activity assays, such as a fluorescence based assay, a reporter gene assay, displacement assays or ELISA.

The new technique also has the potential to allow NMR studies of proteins of much larger size than traditional SAR-BY-NMR since essentially no assignment of the protein resonances has to be undertaken. Conventionally, only proteins of the size up to approximately 30 kDa are possible to study. With the method of the invention, proteins of the size 50 kDa, and most probably of the size up to 100 kDa, can be monitored. The sample preparation is comparatively simple using previously described protocols. [Muchmore]

Now the invention is described by the following example, which only are to be seen as examplifying the invention, and are not to be considered as limiting in any respect.

EXAMPLES

EXAMPLE 1: Screening for binding to the Y-loop of Protein Tyrosine Phosphatase-1B (PTP1B)

PTP1B (35 kD, single domain protein] is a protein that dephosphorylates phosphotyrosines. The active site binding cleft is centered around an active cysteine residue. It has been speculated that the so-called Y-loop of PTP1B is important for binding of some ligands. The Y-loop contains a sequence motif that is unique in the sequence, i.e. Arg47-Asp48 (Fig. 1). A selective labelled sample of PTP1B was prepared to monitor binding to this site.

Site specific labelled PTP1B (residues 1-298) was prepared from transformed *Escherichia coli* strain BL21(DE3) cells. Bacteria were grown in rich medium containing all 20 amino acids and 5 nucleotides according to the protocol described by Muchmore et al. [Muchmore, 1989] Aspartate and arginine were supplied ^{15}N-enriched and ^{13}C-enriched, respectively. It should be noted that this protocol (i.e. the use of a prototrophic bacterial strain) for preparing the sample is not optimal for the production

of selectively ^{15}N-Asp enriched protein. Aminotransferase activity may cause misincorporation of ^{15}N in other amino acid residues. For the case of Asp, incorporation in Asn, Glu and Gln residues may be seen. [Muchmore, 1989] Nevertheless, the risk of misincorporation of the ^{15}N label is in no way a limitation to the invention. A selectively labelled protein may be produced with no misincorporation of the ^{15}N (or ^{13}C) label if a bacterial strain with gene lesions suitable to the particular type of selective amino acid enrichment is used. [Muchmore, 1989] It should also be noted that in favorable cases good results have been reported also when prototrophic bacterial strains were used. [Kainosho, 1982] [Muchmore, 1989]

A two-dimensional ^{1}H-^{13}C correlation spectrum of the selectively labelled sample verified that only Arg residues were ^{13}C-enriched. The corresponding ^{1}H-^{15}N experiment indicated, however, that ^{15}N was not exclusively incorporated in Asp residues. Figure 2A shows the 2D ^{1}H-^{15}N TROSY [Weigelt, 1998] spectrum of the ^{15}N-Asp, ^{13}C-Arg sample. If only Asp residues were ^{15}N-labelled a total of 18 peaks would be expected in this ^{1}H-^{15}N correlation spectrum (18 Asp residues in the amino acid sequence). Instead, the spectrum contains approximately 65 peaks, which is consistent with incorporation of ^{15}N also at Asn, Glu and Gln sites (totally 69 in the amino acid sequence). Studying the amino acid sequence in detail one can see that there now instead of one unique pair, Arg47-Arg48, are four additional ones, Arg43-Asn44, Arg156-Gln157, Arg169-Glu170, and Arg199-Glu200. The first of these, Arg43-Asn44, is located in the beginning of the Y-loop. The other three are situated on the protein surface far from the binding site (Fig. 3).

Figure 2B shows the 2D HNCO [Cavanagh, 1996] spectrum that selects signals from the selectively labelled pairs. As expected, five peaks corresponding to the five unique sequence pairs appear in the spectrum (circled). An additional peak (marked with an arrow) originating from the C-terminal Asp residue is also seen. The natural abundance ^{13}C (1 %) of the preceding Glu residue is enough to yield an HNCO signal due to the extremely strong H^{N}-resonance of the C-terminal Asp residue.

To detect binding to the Y-loop one would expect that one or two of the designated signals would experience large chemical shift changes upon addition of a compound

that interacts with the Y-loop, whereas the other three signals would remain essentially unaffected.

To test this hypothesis the inventors recorded 2D TROSY spectra in the presence of a cocktail of five compounds. One of the compounds, N200, was known to bind to the protein as determined by a NMR-line broadening assay (more than 5 Hz line broadening at 50 μM concentration in the presence of an equimolar amount of PTP1B). Three compounds, N35, N136 and N212, were non-binders as determined by NMR. The fifth compound, PNU179983, was a micromolar inhibitor of PTP1B believed to interact with the Y-loop. Figure 2C shows a selected area from the TROSY spectrum of free ^{15}N-Asp, ^{13}C-Arg PTP1B. Figure 2D shows the spectrum in presence of the cocktail. Figure 2E shows the spectrum in presence of all compounds from the cocktail except PNU179983. Figure 2F shows the spectrum with only PNU179983 present. In all spectra the signals corresponding to the HNCO selected signals are circled.

It is evident that substantial spectral changes occur in the presence of the compound cocktail (Fig. 2D). In particular two of the designated peaks (marked with asterisks) disappear altogether, whereas the other three signals only experience minor shifts. This indicates that at least one of the compounds in the cocktail interacts strongly with the Y-loop. When PNU179983 was omitted from the cocktail (Fig. 2E) no shifts are observed for any of the designated signals. Figure 2F shows that PNU179983 is responsible for the large shifts since this spectrum shows the same pattern as the one recorded in presence of the full cocktail (Figure 2D).

To further substantiate the finding that the invention allows identification of binders to a selected site all compounds were tested in a colorimetric assay (Fig. 4). N 200, which binds to PTP1B according to the NMR line-broadening assay, showed partial inhibition of the enzyme, indicating binding to the active site. N35 and N136 also showed partial inhibition, which would indicate that they were false negatives or interacting too weakly to indicate binding in the NMR line-broadening assay. PNU179983 showed complete inhibition of the enzyme.

Furthermore, an x-ray crystallographic study was performed to assess the binding mode of PNU179983. Preliminary x-ray crystallographic data at 2.2 Å resolution (Derek Ogg, unpublished results) showed that PNU179983 binds to PTP1B with interactions not only within the active site but also with the Y-loop (Fig. 5). The inhibitor is in close proximity to the Y-loop and interacts with the loop via hydrogen bonds. The shortest distance between the inhibitor and the Y-loop is approximately 2.8 Å.

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CLAIMS

1. Method for identifying at least one binder molecule comprising the steps of:
   (a) choosing two amino acid types (AA1 and AA2) in a polypeptide or protein of interest, whereby AA2 at least once occurs directly subsequent to AA1 in the amino acid sequence of the polypeptide or protein, defining an amino acid pair AA1-AA2;
   (b) labelling the two amino acid types (AA1 and AA2) in the polypeptide or protein of interest, whereby all AA1-residues is labelled with ^{13}C and all AA2-residues with ^{15}N;
   (c) generating a first HNCO-type NMR spectrum of the labelled polypeptide or protein from step (b), thereby identifying signals from the labelled amino acid pair AA1-AA2;
   (d) contacting the labelled polypeptide or protein with a potential binder molecule or a mixture of binder molecules under conditions and sufficient time for allowing binding of the potential binder molecule(s) and the labelled polypeptide or protein;
   (e) generating a second HNCO-type NMR spectrum, or a ^{1}H-^{15}N correlation type NMR spectrum, of the mix from step (d), monitoring signals identified in step (c); and
   (f) comparing the first and the second NMR spectra, whereby a chemical shift change of the signals identified in step (c) between the two spectra indicates an interaction between the potential binder molecule and the labelled polypeptide or protein.

2. Method according to claim 1, whereby the labelled amino acid pair AA1-AA2 is unique within a sphere radius of 10 Å, preferably 50 Å, and most preferably within the whole polypeptide or protein.

3. Method according to anyone of claims 1-2, whereby the labelled amino acid pair AA1-AA2 is within a binding pocket of the polypeptide or protein.

4. Method according to anyone of claims 1-2, whereby the labelled amino acid pair AA1-AA2 is in the proximity of an active site within the polypeptide or protein.

5. Method according to anyone of claims 1-2, whereby the result of the method is compared to the result of any other suitable binding or activity assay.

6. Method according to any of the preceding claims, whereby the polypeptide or protein has a size of 10 – 150 kDa.

7. Method according to any of the preceding claims, whereby the potential binder molecule is a peptide, a polypeptide, a protein, an antibody, a nucleic acid molecule, a carbohydrate, or a complex of one or more of the mentioned molecule types The potential binder molecule may be a peptide, a polypeptide, a protein, an antibody, a nucleic acid molecule, a carbohydrate, a part or a complex of one or more of the mentioned molecule types, or any other chemical compound of interest.

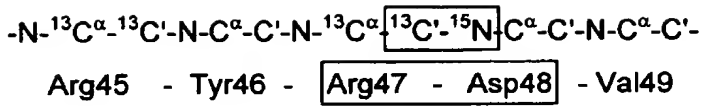
8. Method according to any of the preceding claims, whereby the potential binder molecule is a relatively small molecule, preferably in the size interval of 50-1000 Daltons.

9. Method according to anyone of the preceding claims, whereby the method is used for screening a compound library.

ABSTRACT

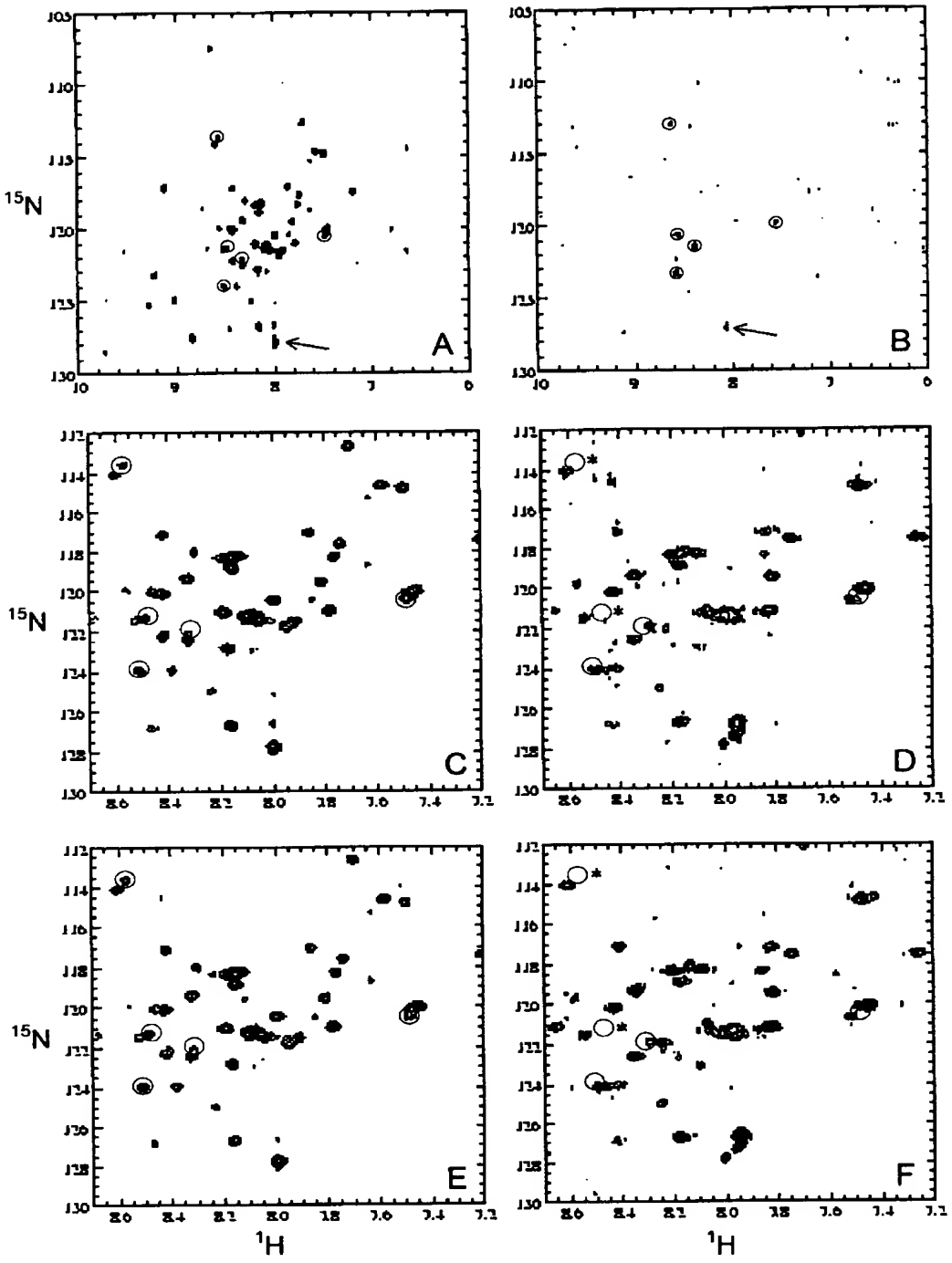
The invention relates to a method for identifying at least one binder molecule comprising the steps of: (a) choosing two amino acid types (AA1 and AA2) in a polypeptide or protein of interest, whereby AA2 at least once occurs directly subsequent to AA1 in the amino acid sequence of the polypeptide or protein, defining an amino acid pair AA1-AA2; (b) labelling the two amino acid types (AA1 and AA2) in the polypeptide or protein of interest, whereby all AA1-residues is labelled with ^{13}C and all AA2-residues with ^{15}N; (c) generating a first HNCO-type NMR spectrum of the labelled polypeptide or protein from step (b), thereby identifying signals from the labelled amino acid pair AA1-AA2; (d) contacting the labelled polypeptide or protein with a potential binder molecule or a mixture of binder molecules under conditions and sufficient time for allowing binding of the potential binder molecule(s) and the labelled polypeptide or protein; (e) generating a second HNCO-type NMR spectrum, or a ^{1}H-^{15}N correlation type NMR spectrum, of the mix from step (d), monitoring signals identified in step (c); (f) comparing the first and the second NMR spectra, whereby a chemical shift change of the signals identified in step (c) between the two spectra indicates an interaction between the potential binder molecule and the labelled polypeptide or protein.

Figure 1.



Site specific labelling of PTP1B. All arginine and aspartate residues have been enriched with ^{13}C and ^{15}N, respectively. There is only one unique Arg-Asp pair in the sequence (boxed).

Figure 2

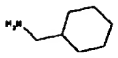
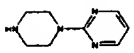
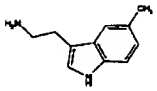
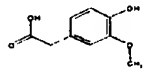
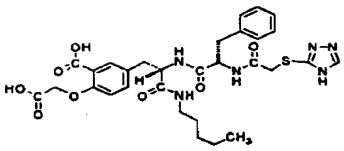


2D NMR spectra of ^{15}N-Asp,^{13}C-Arg labeled PTP1B. A) 2D TROSY of free protein, B) 2D HNCO of free protein, C-F) 2D TROSY spectra of C) free protein, D) protein + compound cocktail, E) protein + compound cocktail omitting PNU179983 and E) protein + PNU179983.

Figure 3

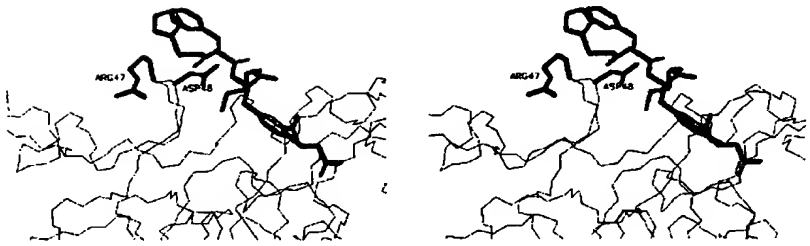
Ribbon drawing of PTP1b showing the five selectively labelled amino acid residue pairs. The "Y-loop pairs" Arg47-Asp48 and Arg43-Asn44 are drawn in red and pink, respectively. Arg156-Gln157, Arg 169-Glu170 and Arg199-Glu200 are all drawn in yellow. The active site cysteine is colored white.

Figure 4

control (no compound added)		0	-
N35		14	no
N136		28	no
N200		20	yes
N212		0	no
PNU179983		100	yes

All compounds were tested in a colorimetric assay. Samples containing 1 μM PTP1B, 2 mM para-nitrophenylphosphate (substrate) and 1 mM compound were prepared in 20 mM Tris-HCl buffer at pH 7.5 and incubated at room temperature for 5 minutes. The reaction was stopped by addition of NaOH to raise the pH. The amount of inhibition was calculated from the measured absorbance of the resulting product (para-nitrophenole).

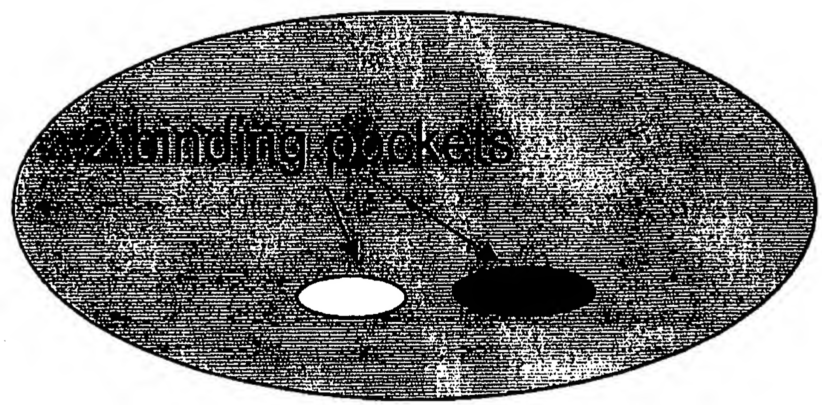
Figure 5



Stereo-view of PNU179983 binding to PTP1B as determined from a preliminary x-ray crystallographic electron density map obtained at 2.2 Å resolution. PNU179983 clearly interact not only with the active residues but also have close contacts with the Y-loop (highlighted) via hydrogen bonds. The closest distance between PNU179983 ant the Y-loop is approximately 2.8 Å. The crystals were grown in the presence of 2 mM inhibitor in 0.1 M pH 6.5 cacodylate buffer, 0.2 M Mg acetate, 16-20 % PEG 8000. The crystals belonged to space group P222 with cell dimensions a=53.2 Å, b=84.3 Å, c=88.7 Å, alpha=beta=gamma=90. Data extended to 2.2 Å with Rsym=6.7%. Data was processed and the model refined to R=25%.

Figure 6

Application I: screen for binding to single binding site

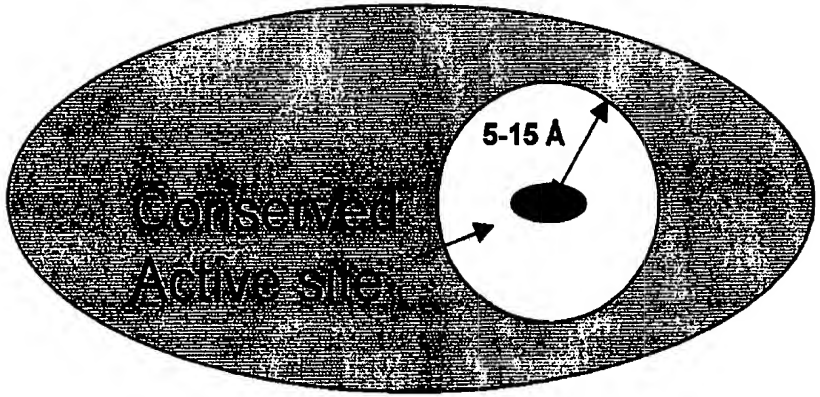


Unique aa pairs in second binding pocket
⇒ use to screen for binders to second pocket

target ...AQSYIEKISQAMESAIEKRLTLAQIMEWIRRNIMG...

Figure 7

Application II: screen for specificity in vicinity of active site



Unique aa pairs in vicinity of active site
only in target sequence ⇒ screen for selectivity

```
target      ...NQSYIELISQAMESAPEKRLTLAQIHEWIRRNAWG...
protein x   ...PYSYISLITMAMQQAPEKMLTLAQIHEWILTHAKP...
protein y   ...PYSYIALITMAMLQSPEKKLTLAQIHEFILVNAKP...
protein z   ...PYSYIELITMAMQNAPEKKITLAQIHQFILVQAKP...
```